The Effect of Parasitism on Personality in a Social Insect

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Abstract

It has been recognised for some years that animals differ consistently in various aspects of their behaviour, a phenomenon that has come to be referred to as animal personality. Recent work has attempted to investigate the ecological factors that shape personality, including the forms of stress that affect its expression. One of these – disease – is known to exert a considerable effect on host behaviour, yet its impact on animal personality has been relatively understudied. This study demonstrates that wood ants, *Formica rufa*, show consistent individual differences in three personality traits: boldness, sociability and aggressiveness; however there was little evidence of substantial correlations between these traits at the group level (known as behavioural syndromes). There was only limited evidence that exposure to the parasitic fungus *Metarhizium anisopliae* had an effect on the mean personality traits, with challenged ants showing marginal changes in boldness and sociability at high doses of fungus but no change in aggressiveness even when close to death. The results suggest that individual personality in *F. rufa* is very resilient to the physiological stress caused by pathogenesis. This may be because, as social insects, higher-order behavioural variation such as caste- and colony-level personality may play a larger role in host-parasite interactions.

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Chapter 1 General Introduction

Summary

The alteration of the behaviour of a host by a parasite has been a source of intrigue for zoologists for decades, and in recent years many researchers have begun to see it as part of the complex, multidimensional nature of host-parasite relationships in which a broad range of host phenotypic traits are affected. Several examples of parasites affecting the behaviour of social insect species have been the subject of recent detailed study, however there has been relatively little attention paid to the unusual aspects of social insect reproductive biology and population structure when considering the evolutionary implications of these observations. It is well known that parasites exert considerable evolutionary pressure on their hosts and can dramatically affect their biology, and the importance of these dynamics suggests that the behavioural effects of parasitic infection may also play a role. In particular, the effects that parasites may have on animal personalities and behavioural syndromes could be significant given that social insects rely on division of labour, in which individuals often display behavioural as well as morphological specialisations for particular tasks. The study of parasite effects on hosts at levels of organisation above the individual organism can draw these disciplines together. The mechanisms by which social insects acquire their different behavioural phenotypes and the influence that parasites may have on this could be a promising direction for future research.

Social insects: a unique model for parasitic manipulation

The study of the effect of parasitism on host behaviour has yielded some exciting revelations and new directions in recent years, from the increased sophistication of experimental techniques and theoretical frameworks used to investigate the neurochemical mechanisms behind such behaviours (Kavaliers, 2000; Hoover *et al*., 2011; Perrot-Minnot & Cézilly, 2013), to the recognition of the multidimensionality of host manipulation by parasites (Thomas *et al*., 2010; Poulin, 2013). Recently there have been efforts made to combine approaches from multiple disciplines in the biological sciences to understand these complex interactions. However, although these processes have been investigated in some detail in many animal taxa, their importance in social insects is still relatively understudied (Hughes, 2005), in spite of the fact that the unique biology of these organisms may provide useful insights into the evolutionary significance of parasitic manipulation.

Social insects, primarily comprising the cooperative species of ants, bees, wasps and termites, have been described as one of the great achievements of evolution (Wilson, 1971). In the "truly" social (eusocial) insects, colonies of individuals are organised in a highly cohesive way, with cooperative brood care, overlapping generations and reproductive division of labour (Wilson, 1971). The existence of this extreme form of altruism posed a challenge for early evolutionists, with Darwin describing the problem as "insuperable, and actually fatal for the whole theory" (Darwin, 1859). The social insects were seen as representing the advantages of group-living, an example of the reproductive benefit created by "mutual aid which they practice at every stage of their busy and laborious lives", and which has allowed them to become "so numerous that the Brazilians pretend that Brazil belongs to the ants, not to men" (Kropotkin, 1902). Indeed, the dominance of social insects is such that in some tropical environments they may account for over half the free-living biomass (Hölldobler & Wilson, 2009). Greater insight into their success was provided by the development of the

theory of kin selection, whereby individuals gain fitness by aiding the reproductive success of their kin (Hamilton, 1964), and was further advanced when the haplodiploid nature of the Hymenoptera (ants, wasps and bees) and its effect on colony sex ratios was considered (Trivers & Hare, 1976). This led to a proliferation of theoretical and experimental work on the role of kin selection in social behaviour.

The scientific and economic importance of social insects has led to intensive study of their genetics and physiology. Furthermore, their complex social organisation has unique implications for questions regarding host-parasite dynamics. Large numbers of closely related individuals living in very dense colonies and relatively isolated from other colonies suggests that disease transmission will be different to that in non-social animals (Schmid-Hempel, 1995; Schmid-Hempel, 1998). This introduction will cover some of the background on the ways host behaviour is altered by disease and some recent examples in the social insects, as well as potential future directions.

Parasites and behaviour: a broadening discipline

The alteration in behaviour exhibited by animals infected with various parasites has been known for some time. Early work on this phenomenon focused on descriptions of the novel behaviours displayed by animals infected by parasites, but the subject has since expanded into a broad discipline (Moore, 2002). The ecology of parasites was understudied for some time, but recently there have been increased efforts to understand the effects of manipulative parasites on ecosystem functioning (Lefèvre *et al.*, 2009). In a recent review, Liberstat *et al.* (2009) detail some of the more striking behaviours that parasites and parasitoids induce in their hosts through sophisticated effects on the central nervous system. In conceptual work, Dawkins (1982) argued that manipulation in infected animals is an example of an extended

phenotype of the parasite; the parasite's genes control the host's behaviour as a way to ensure its own reproduction and transmission. This extended phenotype view may continue to be useful with the increased sophistication and availability of molecular and computational techniques for the study of host-parasite dynamics (Hughes, 2013).

The view that behavioural changes are adaptive came under attack due to the lack of attention given to systematic methods for testing this assumption (Poulin, 1995; Moore & Gotelli, 1990), with critics arguing that such narratives were simply cases of "adaptationist storytelling" (Gould & Lewontin, 1979). Behavioural changes may be adaptations by the parasites to facilitate transmission or they may be adaptations by the hosts to minimise further spread, or indeed they may be nothing but coincidental "boring" byproducts (Dawkins, 1990) of other parasite effects on host biology (such as physiological stress) that also happen to affect behaviour in ways that are not the outcome of a selective process. Thomas *et al*. (2005) argue that the distinction is incredibly hard to test, but if the manipulation increases parasite transmission it is reasonable to assume that natural selection would have had some role in its development. This debate has had the advantage of researchers taking a more nuanced and interdisciplinary approach that seeks to understand how the behavioural consequences of parasitic infection fit into the broader range of effects parasites have on host biology. Very complex or novel behavioural traits in infected animals are more prone to interpretation as parasite adaptations as selection is more likely to have played a role in shaping their complexity and they are unlikely to arise simply by chance as a side-effect of parasite development (Poulin, 2010). However, as seductive as such conclusions are it may be that relatively small indirect neurophysiological effects of parasites could have potentially large behavioural outcomes, for instance by interfering with conserved molecular pathways, and so while the underlying processes are poorly understood these examples are not exempt from rigorous comparative tests to show causality. It appears obvious that changes in behaviour in

intermediate hosts that increase their conspicuousness is the result of an extended phenotype effect as a way for parasites to increase transmission to their final hosts, yet evidence for increased vulnerability of infected hosts to predation is still fairly sparse (Cézilly *et al*., 2010), and so closer investigation of the ecological effects of such phenotypic changes is important. Furthermore, in studies in which naturally rather than experimentally infected animals have been investigated it is difficult to distinguish if a behaviour is the result of parasitic infection or if it is a pre-existing behaviour that increases the likelihood of becoming infected. Therefore the explanatory significance of studies using naturally infected hosts is reduced, and future work will have to use experimental infection techniques in order to show causality (Blanchet *et al*., 2009).

A particularly elegant example of a parasite gene exerting a profound effect on host phenotype can be found in viral infections of moth larvae. Using gene knockouts, Hoover *et al*. (2011) found that a single gene from the baculovirus LdMNPV – *egt*, encoding the hormone-inhibiting enzyme EGT – is responsible for a climbing behaviour of *Lymantria dispar* larvae in which they ascend trees and release new virus particles. While cases like these are undoubtedly exciting, it is becoming increasingly apparent that parasitic manipulation of behaviour often takes place in more subtle ways (Thomas *et al*., 2010). Animal behaviour is highly complex and it may be more difficult to evolve mechanisms to induce a novel behavioural trait than to modulate existing ones, such as the propensity to perform a particular pre-existing behavioural sequence. This could occur as a result of parasite interference with signalling pathways that affect various different behavioural traits (Adamo, 2002). Behavioural effects of viral infection in animals usually appear as a complex combination of several neuronal disturbances (Tomonaga, 2004). Furthermore, there can be considerable variability in behavioural response to manipulation due to genetic and environmental factors (Thomas *et al*., 2011). The recognition of this complexity has opened

up the possibility for a multidimensional approach incorporating neuromodulatory systems (Klein, 2003; Perrot-Minnot & Cézilly, 2013). For instance, parasites are capable of altering the levels of neurotransmitters like serotonin and octopamine (Lafferty & Shaw, 2013). This modulation of neurochemistry can be seen as part of the same strategy as overcoming host immune defences and may have evolved in unison, as it may only be a small evolutionary step from altering host physiology to circumvent the host immune system to concurrently enacting physiological changes that modify host behaviour (Adamo, 2013). These processes could be investigated with genomics, transcriptomics and proteomics (Hughes, 2013).

Another related approach to understand the complex effects of parasites on hosts is to consider parasite manipulation of animal personality and behavioural syndromes. Recent work has demonstrated that animals of many species can show 'personalities': differences between individuals in behavioural traits which are consistent across time or context (Bell, 2007a). Examples can include boldness, sociability and activity (Réale *et al.*, 2007). One individual in a population may be more likely to respond to a given situation in a particular way than another individual, and this difference in behavioural traits remains permanent for an extended amount of time. Individuals may also differ consistently in how they react to a range of environmental contexts such as hunger or predation risk. The set of behaviours that an individual displays in a range of environments is called the behavioural reaction norm (Dingemanse *et al.*, 2010). Thus, if two individuals which typically behave sociably show differing levels of sociability across different environments then they can be said to have different behavioural reaction norms. At the population level, behavioural syndromes are suites of correlated behaviours across individuals, which could also be affected by parasites. An example could be a population in which there is a positive relationship between boldness and activity (Sih *et al.*, 2004; Bell, 2007b). Many previous studies have demonstrated a relationship between infection levels and the expression of a single behavioural trait or a

narrow range of traits. However, recent work now acknowledges that parasites may target a suite of related traits (Poulin, 2013). A parasite inducing a completely novel behaviour combined with a radical modification of morphological phenotype is likely to be rarer than a parasite modulating various behavioural tendencies. This could be at the individual level or may only become visible at the population level. For example, a trophically-transmitted parasite could decrease the consistency of the host response to a stimulus associated with a predator, thus reducing the host's ability to escape and increasing transmission to another host. Alternatively, a parasite could alter the reaction norm of the host so that a behaviour that is normally correlated with an environmental stimulus becomes uncoupled and expressed out of context, which may be disadvantageous for the host but allow increased parasite transmission. Similarly, a parasite uncoupling two related behavioural traits that are dependent on one another could be disadvantageous for the host, for example in cases where both crypsis and selection of an appropriate location for this behaviour are related and the expression of one without the other could lead to increased predation (Poulin, 2013). Examples of these forms of manipulation are beginning to be studied by researchers. Infection with the trematode parasite *Microphallus* was found to alter the correlations between certain behavioural traits of amphipods and decrease the variance in their phototactic behaviour at the population level, which could potentially favour the parasite's transmission by homogenising the personality of the amphipods around an average value that increases their susceptibility to predation by the definitive hosts (Coats *et al.*, 2010). Similarly, trematode-infected fish, *Gobiomorphus cotidianus*, show reduced consistency in their boldness (Hammond-Tooke *et al.*, 2012). Studying the behavioural change of infected animals at the fine scales used in these experiments is likely to become increasingly useful for understanding the many ways in which parasites affect behaviour.

Parasites and behavioural change in social insects

As Hughes (2005) notes, one of the most famous case studies of behavioural manipulation occurs in a social insect. Ants infected with the trematode *Dicrocoelium dendriticum* leave their nests and climb to the tops of blades of grass, where they attach themselves firmly with their mandibles. This exposes them to grazing by sheep, the definitive host of the parasite (Carney, 1969). A comparable case of manipulation involving biting on to vegetation – the "zombie ant" behaviour – has been studied recently: the fungus *Ophiocordyceps unilateralis* causes *Camponotus* ants to fix on to the underside of leaves in a stereotyped "death grip", and a fungal stalk emerges from the ant's head and spores are dispersed (Andersen *et al*., 2009; Fig 1A). The behaviour is associated with high levels of the fungus in the ant's brain (Hughes *et al*., 2011), and experimental modification of the behavioural sequence such as relocating the locations of ants on the leaves caused a reduction in parasite fitness, strongly suggesting that the zombie behaviour is a complex adaptation by the parasite. A similarly striking example is found in nematode-infected ants *Cephalotes atratus*, which show physical changes in the form of red abdomens that look like berries in addition to reduced defence responses, increasing their vulnerability to predation by frugivorous birds, the parasite's definitive host (Yanoviak *et al*., 2008; Fig 1B). Concurrent morphological and behavioural change is also displayed by strepsipteran-infected *Andrena* bees, where physical masculinisation of female bees coincides with the disappearance of the sexual discrepancy in nest emergence behaviour. Parasitised bees emerge from the nest at the same time, whereas in unparasitised bees the females usually emerge considerably later, an alteration that may maximise the time available for parasite development and transmission (Straka *et al*., 2011).

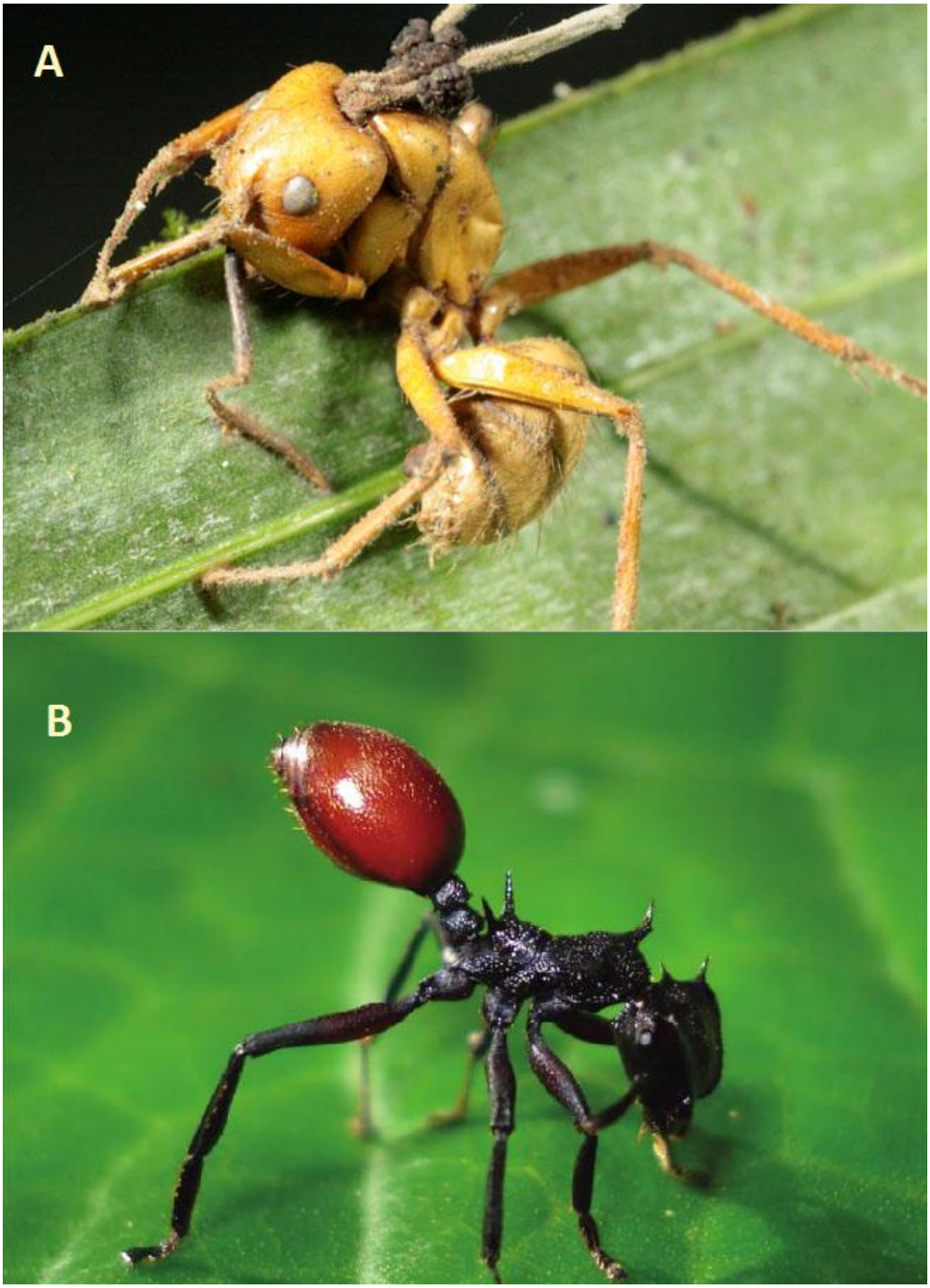


Figure 1 A) is a Brazilian carpenter ant *Camponotus balzani* infected with the fungus *Ophiocordyceps* biting onto a leaf in a characteristic "death grip" while a fungal stalk emerges from the head and sporulates. Photo taken from www.nationalgeographic.com, originally taken by David Hughes.
B) is a parasitised *Cephalotes atratus* worker displaying a swollen and reddened gaster resembling the mature fruit of *Hyeronima alchorneoides*. The ants hold their gasters in a conspicuous position. This behaviour is also associated with a reduction in aggression and defensive behaviours. Photo taken from Yanoviak *et al.* (2008)

The examples discussed so far are very specific, stereotyped behavioural alterations resulting from a parasitic infection, where the adaptive value of the alteration for the parasite is either experimentally demonstrated or at least inferred with some confidence. However, as discussed in the previous section it is becoming increasingly apparent that these sorts of changes, though striking, may be less common than subtler forms of behavioural change. Examples of these include modified foraging behaviour or disrupted cognitive processes. Deformed wing virus (DWV), an extremely prevalent virus of honeybees (Bowen-Walker *et al*., 1999; Evison *et al*., 2012), was found to impair learning and memory in honeybees reacting to an odour stimulus paired with a sucrose reward (Iqbal & Müller, 2007). Infestation with *Varroa destructor* also led to reduced non-associative learning (Kralj *et al*., 2007). The explanation for this is unknown but it may be caused by immune processes triggered by viral infection affecting the molecular mechanisms underlying learning because injection of lipopolysaccharide, a non-pathogenic elicitor of insect immune defences, can also affect associative learning (Mallon *et al*., 2003; Riddell & Mallon, 2006). Similarly, bumblebee workers infected with the protozoan parasite *Crithidium bombi* have an impaired ability to learn the colour of more rewarding flowers (Gegear *et al*., 2006), and those infected with tracheal mites (*Locustacarcus buchneri*) prefer to visit the same types of flowers (Otterstatter *et al*., 2005). Bumblebees found to be infected with *Nosema ceranae* show reduced sensitivity to sucrose (Graystock *et al*., 2013), an observation that may be related to this. An example of a parasite potentially involved in division of labour was found in honeybees, where viral *Kaukugo* RNA was detected in the brains of aggressive workers but not foragers or nurse bees (Fujiyuki *et al*., 2004).

Furthermore, in many cases it is unclear if the alteration is adaptive for parasite or host, or if it is simply a coincidental byproduct of infection. Examples of this include greater foraging recruitment and longer foraging times in bees infested with *Varroa* mites (Janmaat *et al*.,

2000; Kralj & Fuchs, 2006), which may be an adaptation by bees to remove these parasites or may simply be an effect of impaired cognitive abilities due to infection. Similarly, behavioural fever, normally a defence against temperature-sensitive parasites (Wilson-Rich *et al*., 2009), in honeybees infected with the microsporidian *Nosema ceranae* appears to modify the temperature to a level benefitting the parasite – though as a consequence of the energetic stress rather than any direct manipulation – leaving it unclear as to the adaptive value of the alteration to either the parasite or the host (Campbell *et al*., 2010).

One way to start to overcome such difficulties is to investigate the underlying mechanisms of behavioural change in infected hosts, a strategy that is being increasingly made possible with advances in genetics and molecular biology such as quantitative transcriptomics and proteomics (van Houte *et al*., 2013). A recent study found that *Nosema ceranae* and *Varroa destructor* alter the brain transcriptomic profiles of honeybees, though without any actual noticeable behavioural change (McDonnell *et al*., 2013). The fact that both parasites induce a similar effect suggests that the pathways involved are conserved, and future work could investigate whether they have some involvement in either parasite manipulation or host behavioural resistance (Wagoner *et al*., 2013). Analysis of the gene expression of honeybees either resistant or susceptible to *Varroa destructor* showed that the genes with the greatest differential expression were those involved in neuron development, highlighting the importance of the behavioural response to infection as well as the potential impact of *Varroa* on the nervous system of non-resistant hosts. The bee gene *pale* which encodes the enzyme tyramine hydroxylase, involved in dopamine synthesis, is downregulated in infected larvae (Navajas *et al*., 2008). If this is also the case in adults then dopamine-mediated functions including cognition could be impaired. Additionally, two other downregulated genes in *Varroa*-infected bees, *Dlic2* and *Atg18*, are enhancers of the gene *bchs*, which has a role in inhibiting neural degeneration in *Drosophila* (Finley *et al*., 2003). Therefore a lower

enhancement may cause a higher degree of age-related cognitive impairment in infected bees (Navajas *et al*., 2008), though it is not clear that these effects are adaptive for the parasite. Another instance of parasites affecting potential neurogenomic expression was found in *Acromyrmex* ants, where direct inoculation of fungal gardens with *Escovopsis* causes a downregulation in immune-related genes, possibly to allow for neurological processes involved in prophylactic behaviours to be prioritised (Yek *et al*., 2013). These gene expression studies mark just the beginning of the attempt to understand the proximate mechanisms behind the interaction of behaviour and parasitism in social insects.

Social evolution, disease and behaviour

As mentioned above, the social insects are well placed for investigations into the effects of parasites on behaviour. Their ecological dominance and economic importance has led to considerable attention on their biology (Winston, 1987; Hölldobler & Wilson, 1990; Seeley, 1995). Consequently knowledge of their behaviour is relatively well developed, meaning that parasite manipulation is amenable to detailed study (Hughes, 2005). In 2006 the genome of the honeybee was sequenced (The Honeybee Genome Sequencing Consortium, 2006), followed more recently by several ant genomes (Gadau *et al*., 2012; Simola *et al*., 2013), which provides a strong basis to pursue research into the proximate mechanisms of behaviour when combined with neurology (e.g. Menzel & Müller, 1996). The impact of disease on populations of social insects, particularly honeybees and bumblebees, has led to considerable interest in social insect disease defence and immunology (see Cremer *et al*., 2007; Wilson-Rich *et al*., 2009), with investigations into improving these defences (Pérez-Sato *et al*., 2009). It is known that parasites can have a substantial effect on survival of social insect hosts (Schmid-Hempel, 1998; Hughes & Boomsma, 2006; Rutrecht & Brown, 2008; Graystock *et al*., 2013) and contribute towards the widespread losses of honeybee colonies in locations like

the United States (Cox-Foster *et al*., 2007; Higes *et al*., 2008), and it could be that behavioural effects of infection as well as physical stress play a role in this.

Furthermore, the range of defences that social insects have developed to protect themselves against disease is evidence that disease has been an important factor in social evolution (Schmid-Hempel, 1998; Schmid-Hempel, 2005). In evolutionary terms, parasite-mediated negative frequency-dependent selection may act in the opposite direction to kin selection (Shykoff & Schmid-Hempel, 1991a,b). Social insect colonies, as collections of closely related individuals, are potentially more susceptible to parasitic infection, and features that increase the genetic diversity of colonies may in part be adaptations to avoid this (Schmid-Hempel, 1998). For example, multiply-mated bumblebee offspring had lower parasite intensity and prevalence than singly-mated offspring (Baer & Schmid-Hempel, 1999). In honeybees, colonies with queens inseminated by multiple drones had lower disease intensity and greater colony strength than those with queens inseminated by a single drone (Tarpy, 2003; Seeley & Tarpy, 2007), and ants with greater genetic diversity were more resistance to low doses of entomopathogenic fungi (Hughes & Boomsma, 2004). Fungi transmitted through genetically diverse ants were also more likely to be eliminated from the ant populations under experimental conditions, suggesting that parasites may be less able to adapt to more diverse hosts (Hughes & Boomsma, 2006). These results show that parasites have presented a strong enough selective pressure to somewhat counteract the genetically homogenous colony structure favoured by kin selection.

Given the prevalence of social insect parasites and the impact they appear to have made on social evolution and population structure, it seems likely that behavioural manipulation would have comprised a part of this influence and therefore research into this area may highlight important aspects of evolutionary biology. Parasites, like some endosymbionts, may alter the sex ratios of social insect colonies. Infection may reduce dominance behaviours and stop

workers competing with the queen and laying their own eggs, making the colony more cooperative (Shykoff & Schmid-Hempel, 1991c). The threat of disease may favour more social colonies as sociality has been linked to disease resistance (Stow *et al*., 2007). It is known that parasites display context-dependent virulence (Brown *et al*., 2000) and low virulence infection can still have dramatic effects on host biology (Brown *et al*., 2003). Even non-parasitic microbes may alter host behaviour (Ezenwa *et al*., 2012). If small factors such as these can have a large impact on social insect evolution it seems reasonable to assume that the behavioural effects will play at least some role too, yet this has been relatively understudied. Therefore, what questions can be asked about the implications of parasitic manipulation of behaviour in social insects?

Future directions and prospects

Any understanding of the evolutionary biology of social insects must consider the reproductive division of labour central to their nature. It has been argued that colonies, with their members cooperating to form a highly organised structure, represent a 'superorganism' analogous to the cooperation between cells to form an individual organism (Hölldobler & Wilson, 2009). In this view the queens, as the primary reproductive individuals, are like the germ cells of the superorganism and the non-reproductive workers are the somatic cells, which are not involved in its reproduction but help to maintain conditions so that the queen can reproduce. While it is important not to push this analogy too far because various social conflicts exist between members of the same colony in many social species, it is clear that in many situations colonies behave more or less as a unified whole. This has led Hughes (2012) to argue that in addition to individual-level behaviour, parasites may be able to manipulate the behaviour of the superorganism, a view that may be particularly useful in very social species with advanced division of labour in which highly differentiated castes could be

targeted by specific parasites rather like they target particular organs or tissues in individuals (Hölldobler, 2012). In much the same way as behaviour is studied in the context of sociobiology, it could also be discussed in the context of socioparasitology, focusing on the

Box 1 Potential effects on caste- and colony-level behaviour by social insect parasites

Most work on parasitic manipulation is yet to consider host personality and behavioural syndromes, and likewise much of the work on behavioural syndromes addresses populations but not other sub-population groupings such as castes and colonies. Figures I and II below represent some – but by no means all – examples of the sociobiological effects that might occur due to parasitic infection.

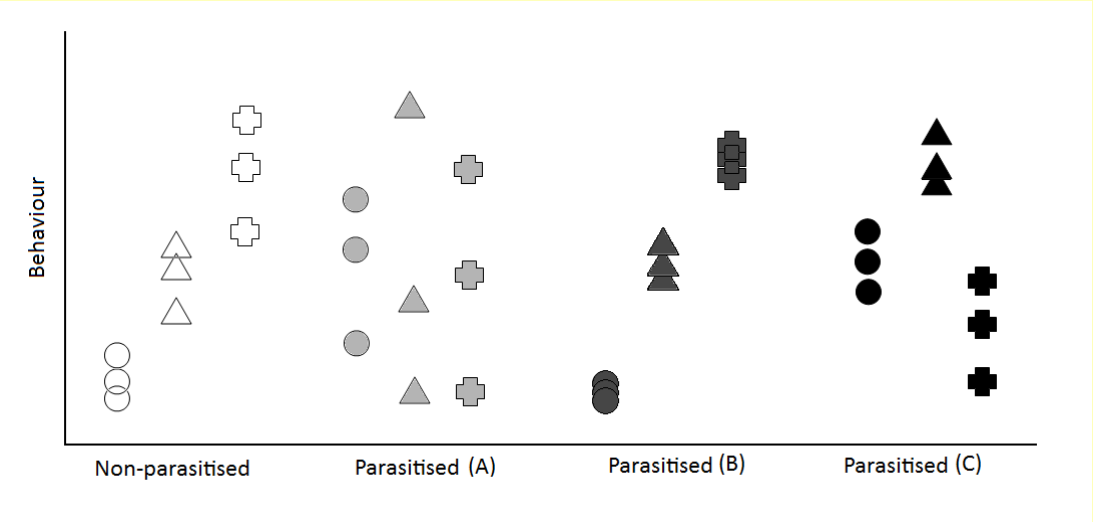


Figure I Average behavioural measurements (e.g. aggressiveness) of different individuals (represented by each symbol) within different castes (each type of symbol is a different caste, for instance circles could be brood carers, triangles could be foragers and crosses could be soldiers) in a social insect colony. Open symbols are non-parasitised and filled symbols are parasitised by three different hypothetical parasites; A, B and C. In the non-parasitised example the variation within castes is less than the variation within the entire colony, thus the value of the behavioural trait is more-or-less associated with caste.
Parasite A destroys this association, so that variation within castes is similar to the total variation within the colony and there are no caste-level behavioural phenotypes.
Parasite B strengthens this association, so that each individual within a caste has a roughly identical behavioural measurement.
Parasite C maintains a similar level of within-caste variation, but the average behavioural measurement for each caste is now altered so that each caste now behaves differently.

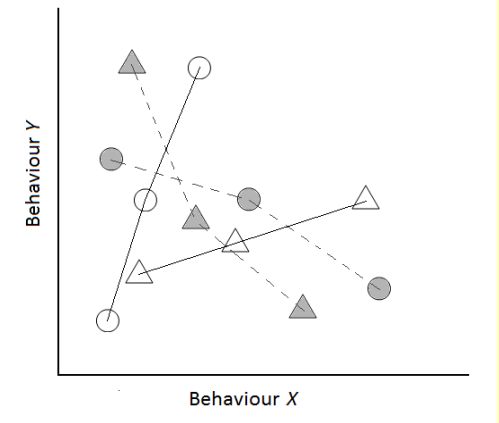


Figure II Correlations in two different behaviours, *X* and *Y*, in social insect host with division of labour into two castes (represented by either triangles or circles) when unparasitised (open symbols) and parasitised (filled symbols). In both cases the direction of the relationship is the same at the caste- and colony-level but the strength of the correlation varies between castes. The effect of the parasite is to reverse this correlation, altering the behavioural syndrome of the host at both the caste and colony level.

Table 1 The within-colony and between-colony variation in behaviour (personality and behavioural syndromes) investigated in social insect species in a sample of the recent literature

Species	Behavioural traits tested	Between-/within-colony	Reference
Honeybee *Apis mellifera*	House-hunting behaviour (scouting activity, waggle dance behaviour)	Within	Wray & Seeley (2011)
Honeybee *Apis mellifera*	Foraging activity, defensive response, undertaking	Between	Wray *et al.* (2012)
Bumblebee *Bombus terrestris*	Response to novel stimuli (neophilia)	Within	Muller *et al.* (2010)
Bumblebee *Bombus terrestris*	Foraging behaviour, response to predation	Within	Muller (2012)
Black harvester ant *Messor andrei*	Foraging behaviour, response to disturbance	Between	Pinter-Wollman *et al.* (2012)
Red ant *Myrmica rubra*	Phototaxis, exploration, activity, curiosity, foraging behaviour, brood care, aggression	Within	Pamminger *et al*. (2014)
Myrmica ant species	Activity, boldness, aggression, sociability, response to alarm pheromone	Within	Chapman *et al*. (2011)
Harvester ant *Pogonomyrmex barbatus*	Foraging activity, emergence	Between	Gordon *et al.* (2011)
Acorn ant *Temnothorax longispinosus*	Aggression, exploration	Within and between	Modlmeier & Foitzik (2011)
Acorn ant *Temnothorax longispinosus*	Aggression, exploration, brood care	Within and between	Modlmeier *et al*. (2012a)
Acorn ant *Temnothorax nylanderi*	Aggression, nest relocation, nest reconstruction, undertaking	Between	Scharf *et al.* (2012)
Acorn ant *Temnothorax rugatulus*	Foraging behaviour, response to novel resource, response to intruder, activity, aggression	Between	Bengston & Dornhaus (2014)

alteration of host behaviour at levels of organisation above the individual. The socioparasitology approach may be helpful when considering the possibilities for future research on the parasitic manipulation of animal personality and behavioural syndromes as discussed by Poulin (2013). In colonies that behave as single superorganisms it seems reasonable to expect that consistent differences in the behaviour of different colonies might be found, and indeed various studies have demonstrated the presence of both individual- and

colony-level personality and behavioural syndromes in social insects (Table 1). In addition to the colony, the fact that many species show distinct morphological differences between different castes, with each caste responsible for a different role within the colony and with a correspondingly specialised behaviour (e.g. foragers, guards, brood-rearers etc), suggests that certain personalities would be favourable for particular castes and so behavioural syndromes would occur at the caste level, as has now been documented (Chapman *et al*., 2011). These additional levels of behaviour increase the complexity of the potential effect of disease. Box 1 suggests a few of the potential ways in which parasites could affect social insect personality. The examples are highly speculative, and given that previous research has largely focused on narrow ranges of host traits there is little evidence that they exist in any real-life species or are even particularly likely. However various patterns of these kinds could be detectable and future studies could design methodologies to test for them. Parasites may have an effect even at low infection levels because single individuals can have disproportionate effects on group-level behavioural traits (Pruitt *et al*., 2013). One can imagine possible parasite advantages from such effects, such as increased inter-colony transmission by increasing the boldness of foragers.

Given that parasites are known to have substantial effects on the behaviour of many social insect species, and have been shown in recent work to influence the personality of several non-social animals, the study of parasite effects on the complex dynamics of social insect personality appears to be a fertile direction for research. The purpose of the study in this thesis is to investigate the potential effects of a parasite on individual-level personality in a social insect. Future work could delve into potential higher-order personality change by comparing the behavioural syndromes of non-infected and experimentally-infected colonies.

Chapter 2 The effect of parasitism on personality in a social insect

Introduction

Recent work in animal behaviour suggests that individual differences in behaviour amongst populations of animals are not simply "noise", but in fact represent consistent tendencies that are analogous to personalities in humans (Bell, 2007a). While it was understood by researchers for some time that higher animals varied in their behaviour (Bell, 2007b), the presence of these differences in less complex animals is now appreciated (Kralj-Fišer & Schuett, 2014), and the literature on their ecological and evolutionary significance has expanded considerably in recent years (Sih & Bell, 2008). Animal personality can be defined as consistent individual differences in behaviour across time and contexts (Bell, 2007b). Examples can include boldness, sociability and aggressiveness. One individual in a population may be more likely to react aggressively in a given situation than another individual, and this difference remains permanent for an extended amount of time. Such polymorphism may be adaptive, for example in species that face a trade-off between risk-prone and risk-averse reproductive strategies (Wolf *et al*., 2007). Individuals may also differ consistently in how they react to a range of environmental contexts such as hunger or predation risk (Dingemanse *et al*., 2010). At the population level, behavioural syndromes are suites of correlated behaviours between individuals. An example could be a population in which there is a positive relationship between boldness, activity and aggressiveness (Sih *et al*., 2004; Bell, 2007b). The position of any given member of the population on the behavioural syndrome is known as that individual's behavioural phenotype.

The growing body of research on animal personality and behavioural syndromes has led to interest in how they are developed and maintained and the various factors that can affect them (Bell & Stamps, 2004; Bell & Sih, 2007). There is evidence that environmental conditions can have an important influence on animal personality. In ants the behavioural variation between colonies is influenced at least in part by weather conditions and nest site (Pinter-Wollman *et al.*, 2012). The range of stressors encountered by animals is known to trigger a wide range of physiological and behavioural characteristics to allow them to cope (Koolhaas *et al*., 1999). Beetles reared on low-quality food are bolder than those raised on a more nutritious diet, which may be a way of increasing short-term reproductive success at the expense of increased threat from predation, compensating for reduced lifespan (Tremmel & Müller, 2013). In general, stimuli that may have adverse effects on fitness, such as reduced availability of food, climatic conditions like low temperatures and increased competition, can trigger behavioural responses (Danchin *et al*., 2008). For example, cold can have serious physiological consequences which could affect behaviour (Modlmeier *et al*., 2012b). Activation of the immune system is known to trigger considerable stress (Moret & Schmid-Hempel, 2000; Korner & Schmid-Hempel, 2004). Honeybees, commonly used in studies of immune responses, have inhibited learning abilities when their immune system is artificially triggered with non-pathogenic lipopolysaccharide (Mallon *et al.*, 2003; Riddell & Mallon, 2006). LPS also causes altered social behaviour that may be a form of behavioural defence against disease (Richard *et al.*, 2008; Alaux *et al*., 2012), and similar behavioural change is found in ants (Aubert & Richard, 2008; De Souza *et al*., 2008).

One of the most injurious forms of stress that animals can encounter is disease, which takes its toll not only through the metabolic investment in immune defences but also through the damage inflicted by the parasite in its attempt to multiply within the host. As such, it is to be expected that parasites would have a dramatic impact on animal behaviour. Perhaps the most

striking way in which disease can influence animal behaviour is parasitic manipulation (Moore, 2002; Hughes, 2005), whereby the parasite induces a specific, stereotyped behaviour in the host that will maximise its own transmission. However, parasitic manipulation of behaviour often takes place in more subtle ways (Thomas *et al*., 2010). Animal behaviour is highly complex and it may be more difficult to evolve mechanisms to induce a novel behavioural trait than to modulate existing ones, such as the propensity to perform a particular pre-existing behavioural sequence. This could occur as a result of parasite interference with signalling pathways that affect various different behavioural traits (Adamo, 2002). Furthermore, there can be considerable variability in behavioural response to manipulation due to genetic and environmental factors (Thomas *et al*., 2011). Instead parasites may target a suite of related traits, meaning that behavioural syndromes can potentially be influenced by disease (Poulin, 2013), and evidence for this is beginning to emerge from studies on fish (Hammond-Tooke *et al*., 2012; Kekäläinen *et al*., 2014) and amphipods (Coats *et al.*, 2010). Another example is the modification of human behaviour by *Toxoplasma* (Flegr, 2013). Instances of parasites affecting host personality and behavioural syndromes need not be adaptive for the parasites could instead be coincidental by-products of other physiological consequences of infection, as is the case in other examples of behavioural change resulting from infection (Poulin, 1995; Moore & Gotelli, 1990). Alternatively, the effects on behaviour could be examples of host behavioural defences against disease, which are especially important in social insects.

The complex social organisation of insect societies has unique implications for questions regarding behavioural variation and also host-parasite dynamics. Consistent differences in behaviour between members of the same colony is expected in social insect species given both their reproductive division of labour and the division of workers into specialised castes which are more disposed to perform certain behaviours within the colony (Dall *et al*., 2012);

however even within these divisions there is individual variation, and recently there is evidence that behavioural variation also extends to the level of caste and colony (reviewed most recently in Jandt *et al*., 2013; see also Table 1 in the previous chapter), highlighting the complexity of these phenomena in social species. The uniqueness of social insects as a model system has led to numerous studies beginning to touch on social insect personality and behavioural syndromes (Kralj-Fišer & Schuett, 2014). For example, bumblebee foragers exhibit consistency in their response to novel stimuli (Muller *et al*., 2010) and swarming honeybees display consistent differences in behaviours related to locating a new nest site such as number of waggle dances and scouting activity (Wray & Seeley, 2011), while *Myrmica* and *Temnothorax* ants vary in many personality traits that are correlated at the caste and colony level (Chapman *et al*., 2011; Bengston & Dornhaus, 2014). Colonies themselves can display consistent variation in behaviour (Gordon *et al*., 2011), and this variation at higher levels of organisation can have adaptive value; for instance various colony level personality traits are associated with fitness of honeybee colonies (Wray *et al*., 2011) and *Temnothorax* ants (Modlmeier & Foitzik, 2011), and are driven in part by intra-colonial variation in behaviour (Pinter-Wollman, 2012; Pamminger *et al*., 2014), demonstrating that individual personality and the way it is structured into colony personality is important for understanding the ecology of social insects.

In addition to raising novel questions about behaviour, social insects are also particularly interesting from the perspective of disease dynamics and host-parasite coevolution. Large numbers of closely related individuals living in very dense colonies and relatively isolated from other colonies suggests that disease transmission will be different to that in non-social animals (Schmid-Hempel, 1995; Schmid-Hempel, 1998). The low genetic diversity between individuals within a colony makes them more susceptible to disease: more genetically diverse ants are more resistance to low doses of the fungus *Metarhizium anisopliae* (Hughes &

Boomsma, 2004) and fungi transmitted through genetically diverse ants are more likely to be eliminated from the ant populations under experimental conditions (Hughes & Boomsma, 2006). Thus parasites have abundant opportunities to spread once inside a colony, but this is usually the more difficult step to achieve (Schmid-Hempel, 1998). Consequently social insects have developed many behavioural defences such as social immunity (Cremer *et al*., 2007; Walker & Hughes, 2009; Wilson-Rich *et al*., 2009), leading to substantial coevolution of host defences and parasite adaptations to evade defences. Therefore behavioural changes in infected insects may result from a complex interplay of parasitic manipulation to increase transmission and insect behavioural responses to reduce transmission.

The importance of infection as a threat to social insect colonies, in addition to the prevalence of case studies demonstrating significant effects of parasitic infection on animal behaviour, suggests that social insect personality may also be affected by disease. Likewise, the variation in personality within colonies is likely to have consequences for parasite effects on behaviour and makes social species uniquely interesting for studying these processes. This study investigates whether infection by the fungal parasite *Metarhizium anisopliae* affects the personality of wood ants, *Formica rufa*, focusing on three personality traits: boldness, sociability and aggressiveness. In the main experiment, we first confirm that the ants show personalities, and then we test the general hypothesis that parasite infection alters the personality of individual wood ants. In two smaller experiments, by focusing on one personality trait (aggressiveness), we test the hypotheses that parasite effects on behaviour are related to the stage of infection and that the effect of the parasite on behaviour is greater than other forms of physiological stress.

Materials and methods

Ant colony maintenance

A queenless subcolony was collected from each of four colonies of *Formica rufa* wood ants in Abbots Wood, East Sussex, UK in August 2013 (colonies FR1 and FR4) and June 2014 (colonies FR3 and FR5). The colonies were kept in plastic boxes containing chipped wood bark at 23±1°C temperature and 35±10% relative humidity on a diet of *Tenebrio molitor* mealworm larvae twice a week, with water and 10% sucrose solution provided *ad libitum*.

Experiment 1: Personality and behavioural syndromes experiment

Individual wood ants were taken from colonies FR1 and FR4 at random and placed individually in lidded plastic pots approximately 30 x 30 x 70mm in size with air holes. Each individual was kept in isolation in its pot for the duration of its use in the experiment. Each ant was tested five times in each of three behavioural assays (boldness, sociability, aggressiveness; see below). The ant was then exposed to either a pathogen challenge or control treatment, left for 48 h and then tested five times in each of the behavioural assays again.

Pathogen challenge

After the first set of assays was conducted each individual was either treated with the *Metarhizium anisopliae* parasite or control solution. *M. anisopliae* is a generalist entomopathogenic species of fungus that is prevalent in the soil environment of ants in many areas and has been reported naturally infecting ants on numerous occasions (Schmid-Hempel

1998; Hughes *et al*. 2004; de Zarzuela *et al*., 2012). It is commonly used in experiments on ant disease (Rosengaus *et al*., 1998; Hughes & Boomsma, 2004). Conidia of the parasite germinate on the ant's surface and produce hyphae that penetrate the cuticle and proliferate within the haemocoel of the insect (Hajek & Leger, 1994; Gillespie *et al*., 2000; Santi *et al.*, 2010). The fungus is highly virulent and eventually kills the host, after which the fungus sporulates (Zhang *et al*., 2010). Suspensions of fungal conidia from the isolate Mt02-73 were obtained from freshly sporulating media plates. The concentrations of the suspensions were determined using FastRead Counting Chambers, and once known they were diluted to the preferred concentrations with 0.05% Triton X solution. Viability of conidia was checked by plating the suspensions onto Sabaroud dextrose agar media plates and counting the proportion of germinating spores after incubating at 32°C for 24 hours.

Ants were treated by applying 1µL of the conidia suspension to the outside of its thorax and abdomen, or treated with 1µL 0.05% Triton X solution as a control. Individuals were then returned to their plastic pots and provided with cotton wool soaked in 10% sucrose solution. The behavioural assays were conducted again on the same individuals 48 h later to allow comparison of the 'before' and 'after' personalities.

The experiment was carried out twice with different concentrations of fungal suspension. In **Experiment 1A** treated ants were challenged with a low dose (1×10^7 conidia/ml) suspension (N = 17 individuals). In **Experiment 1B** a high dose (1×10^8 conidia/ml) suspension was used (N = 30 individuals).

Behavioural assays

Three separate assays were designed to quantify three behavioural traits commonly examined in the animal personality literature: boldness, sociability and aggressiveness.

Boldness of individual *Formica rufa* was quantified by measuring the amount of time each individual took to emerge from a sheltered area into a more exposed environment. The focal individual was placed into a 45mm petri dish, darkened inside with a red acetate film covering the top and black electrical tape covering the sides, and with a $1mm^2$ opening made in the side which was obstructed by a door of paper coated in electrical tape. The individual was allowed 2 mins to acclimatise and the door was removed. The length of time before the ant fully emerged from the petri dish was measured with a stopwatch. A cut-off of 15 mins was used, after which it was assumed the individual would not emerge and the data point was discounted.

Sociability was quantified by measuring the amount of time an individual associated in close contact with a member of the same colony. The focal individual was placed into a 245mm x 185mm x 75mm clear plastic box with $1cm^2$ gridded paper underneath containing another individual selected at random from the same colony. The ants were recorded for 5 mins using a webcam and the videos were analysed to calculate the proportion of time the ants associated within 20mm of each other (a distance used by Chapman *et al.*, 2011).

Aggressiveness was quantified by measuring the number of aggressive responses to a conspecific of a different colony using the mandible opening response technique, which has been effectively used to test defensive behaviours in other ant species (Guerrieri & d'Ettorre, 2008; Norman *et al*., 2014). The focal individual was immobilised with carbon dioxide and placed into a harness, made from an Eppendorf tube with the bottom cut off, and secured with a thin strip of masking tape so that only the head and antennae were free. The ant was allowed to acclimatise and then presented with a stimulus of a freeze-killed ant from a different colony. Each presentation of the stimulus was separated by 10 min intervals. Each stimulus was presented to the ant so that its antennae were allowed to touch it for 10 s. The ant opening its mandibles for >1 s was counted as a positive (aggressive) response. The

number of positive responses to the non-nestmate was used to determine the individual's aggressiveness.

Individuals were returned to their pots in between assays and allowed to acclimatise before being transferred to the next assay. Each individual was tested in each assay five times during a single day and an average of the repeats was calculated. The assays were repeated in a random order except the aggressiveness assay, which was conducted last for all individuals to eliminate the need to expose the ants to carbon dioxide multiple times.

Experiment 2: Fine time-scale experiment

In order to investigate the behavioural effects of *Metarhizium* at a finer time scale, the average behaviour of *Formica rufa* was investigated at 12 h intervals to determine whether any change is more likely to occur at certain stages of infection.

As in Experiment 1, individuals were selected at random from the colonies, this time from the more recently collected colonies FR3 and FR5, and isolated in plastic pots containing cotton wool soaked in sucrose solution. The ants were treated with *Metarhizium* in the manner described in Experiment 1, or left untreated. 20 individuals were transferred to the behavioural assay immediately for the '0 h' group and were not challenged with the parasite.

In this experiment only the aggressiveness of the ants were tested using the mandible-opening response (MOR) assay described in Experiment 1 because it was the least time-intensive assay and therefore allowed larger sample sizes and more efficient data collection. Individuals were presented with a freeze-killed ant from another colony and the presence or lack of a positive (aggressive) mandible opening response was recorded. This was repeated five times for each individual, with a gap of at least 10 mins in between stimuli, and the total

number of positive MORs out of a possible five was used to determine the ants' aggressiveness. Assays were conducted at 12 h intervals between 8.00 and 21.00 up to 72 h from the time of treatment. The procedure was repeated once on consecutive weeks to yield a total of 20 ants at each time period, 10 treated and 10 control, except the first 0 h time period in which no ants were challenged (total sample size after mortality = 150).

Experiment 3: Physiological stress experiment

In order to investigate whether any other kinds of physiological stress (in addition to that caused by isolating individual ants in plastic pots for a number of days) have an effect on ant behaviour, and whether pathogen challenge has a greater effect than these other stressors, the behaviour of *Formica rufa* was investigated after exposure to various external and internal conditions.

Individuals were selected at random from colonies FR3 and FR5 and isolated in plastic pots containing cotton wool soaked in 10% sucrose solution. Individuals were then tested in a behavioural assay. As with Experiment 2, only the aggressiveness assay from Experiment 1 was used. Individuals were presented with a freeze-killed ant from another colony and this was repeated five times for each individual. The total number of positive MORs out of a possible five was used to determine each ant's aggressiveness. The ants were then exposed to five treatments: 1) cold shock, 2) stimulation of the immune system, 3) stimulation of oxidative stress, 4) challenge with the *Metarhizium* parasite and 5) a control. They were then returned to their pots and left for 48 h, after which time the aggressiveness assay was repeated.

To subject ants to cold shock they were placed in a freezer at -18°C for 3 mins. To stimulate the ants' innate immunity the ants were injected with lipopolysaccharide (LPS; Sigma L-2755), a cell membrane surface polysaccharide isolated from *Escherichia coli* which is known to elicit certain immune pathways in invertebrates without causing pathogenesis (Moret & Schmid-Hempel, 2000; Mallon *et al*., 2003). LPS was dissolved in Ringer's solution to a concentration of 0.5mg/ml, a concentration used in previous studies using LPS (e.g. Korner & Schmid-Hempel, 2004; Richard *et al*., 2008). To stimulate oxidative stress, ants were injected with paraquat dichloride (Sigma 36541), a pneumotoxicant which is known to induce the formation of reactive oxygen species in invertebrates (Seehuus *et al*., 2005) and trigger an injurious effect on cells (Day *et al.*, 1999). A concentration of 1mg/ml in Ringer's solution was used because when adjusted for the difference in body mass between honeybees (*Apis mellifera*) and *Formica rufa* (average body mass in Experiment 1 = 12.3mg) this is similar to the dose used by Seehuus *et al*. (2005) to control for the confounding effect of social stress when the insects were removed from the colony. To challenge the ants with the *Metarhizium* parasite, they were treated with a 1 x 10^8 conidia/ml suspension in the manner described in Experiment 1. As a control, ants were injected with Ringer's solution because this isotonic solution should not cause stress to the ants on its own and controlled for the effect of injection in the LPS and oxidative stress treatment groups. Injections were performed similarly to Amdam *et al*. (2003) with a micro-syringe (Hamilton) between the second and third tergites of the ants' abdomens, the ants having been immobilised with carbon dioxide and secured in an Ependorff tube in a manner similar to that used for the MOR assay but with the abdomen exposed rather than the head. The injection volume was 0.5μL. A total of 180 individuals were treated, but the ants suffered some mortality between trials and this left a total sample size of 116 individuals for which data for both the 'before' and 'after' trials could be collected.

Statistical analysis

For Experiment 1 the results of each assay were analysed with a generalized linear model (boldness and sociability: gamma error distribution with log link function; aggressiveness: binomial distribution with logit function) to test for an effect of treatment as well as the interaction of individual personality differences and any differences between the 'before' and 'after' trials. The correlation between the average 'before' and 'after' personality traits in both the treatment and control groups were analysed for each personality trait using Spearman rank correlation tests. In addition, a GLM regression analysis, with the 'before' value as a covariate of the 'after' value, was performed for each trait to test whether these associations were different between the treatment and control groups.

For Experiments 2 and 3 the results were analysed with generalized linear models (binomial error distribution with logit link function) to test for an effect of the length of time since treatment as well as an interaction of any effect of treatment with time in Experiment 2, and to test for individual personality differences as well as the interaction of any effect of treatment and any differences in the 'before' and 'after' trials in Experiment 3.

Statistics were carried out using SPSS 21.

Results

Experiment 1: Personality and behavioural syndromes experiment

In both Experiment 1A (low dose) and Experiment 1B (high dose) there were significant differences between individuals in their boldness (χ^2 = 301, df = 20, P < 0.001; χ^2 = 408, df = 28, P < 0.001, respectively), sociability (χ^2 = 39.2, df = 19, P = 0.004; χ^2 = 81.5, df = 28, P < 0.001) and aggressiveness (χ^2 = 37.7, df = 19, P = 0.006; χ^2 = 135, df = 28, P < 0.001). Individuals varied by as much as 1200-fold in their boldness, whilst their sociability varied between no interaction at all with a nestmate or constant association with a nestmate and their aggressiveness varied between an aggressive response to all stimuli or no response at all (Figs. 1 and 2). However, there was little evidence of relationships between the boldness, sociability and aggressiveness of individuals. The correlation between boldness and sociability was significant but weak (ρ = 0.382, N = 51, P = 0.006), and the correlations between boldness and aggressiveness or sociability and aggressiveness were both non-significant (ρ = -0.138, N = 51, P = 0.334, and ρ = -0.068, N = 51, P = 0.634, respectively; Fig. 3).

The effect of treatment with *Metarhizium* on personality was not clear-cut; many individuals showed considerable change in their behaviour between the 'before' and 'after' trials, although there was no obvious difference between the treatment and control groups (Figs. 1 and 2). There were significant interactions between treatment and trial in the boldness assays at both the low and high doses (χ^2 = 109, df = 1, P < 0.001; χ^2 = 193, df = 1, P < 0.001, respectively), indicating that the differences in the time individuals took to emerge in the 'before' and 'after' trials differed significantly between the treatment and control groups. In the sociability assay this interaction was not significant in the low dose experiment (χ^2 =

1.202, df = 1, $P = 0.273$) but was significant in the high dose experiment ($\chi^2 = 7.27$, df = 1, $P = 0.007$), whereas there was no significant interaction in the aggressiveness assay in either experiment ($\chi^2 = 1.99$, df = 1, $P = 0.159$; $\chi^2 = 0.367$, df = 1, $P = 0.544$).

The effect of treatment could also be investigated by comparing the correlations between the 'before' and 'after' personality traits of the ants in the different treatment groups. In general the relationships between the 'before' and 'after' values for the three personality traits were quite weak (Fig. 4); the only significant correlations were between the treatment aggressiveness values in the low dose experiment ($\rho = -0.871$, N = 10, $P = 0.001$) and the control group boldness values in the high dose experiment ($\rho = 0.744$, N = 16, $P = 0.001$), and the GLM regression analysis showed that the slopes of the associations between the treatment and control groups in both cases were significantly different to each other ($\chi^2 = 11.2$, df = 1, $P = 0.001$; $\chi^2 = 15.2$, df = 1, $P < 0.001$). Furthermore, the direction and magnitude of change remained relatively similar between the treatment groups, with both the treatment and control groups containing individuals that experienced small and large changes in their personality traits between trials (Figs. 5 and 6). By classifying each change value as positive or negative, the chi-squared test (or Fisher's exact test in cases where the assumptions of chi-squared could not be met) was used to test for differences in the direction of change between the treatment groups. Of the behavioural traits there was only a significant difference in sociability in the high dose experiment, in which four of the individuals became more sociable and ten became less sociable in the treatment group whereas twelve individuals became more sociable and four became less sociable in the control group ($\chi^2 = 6.47$, df = 1, $P = 0.011$).

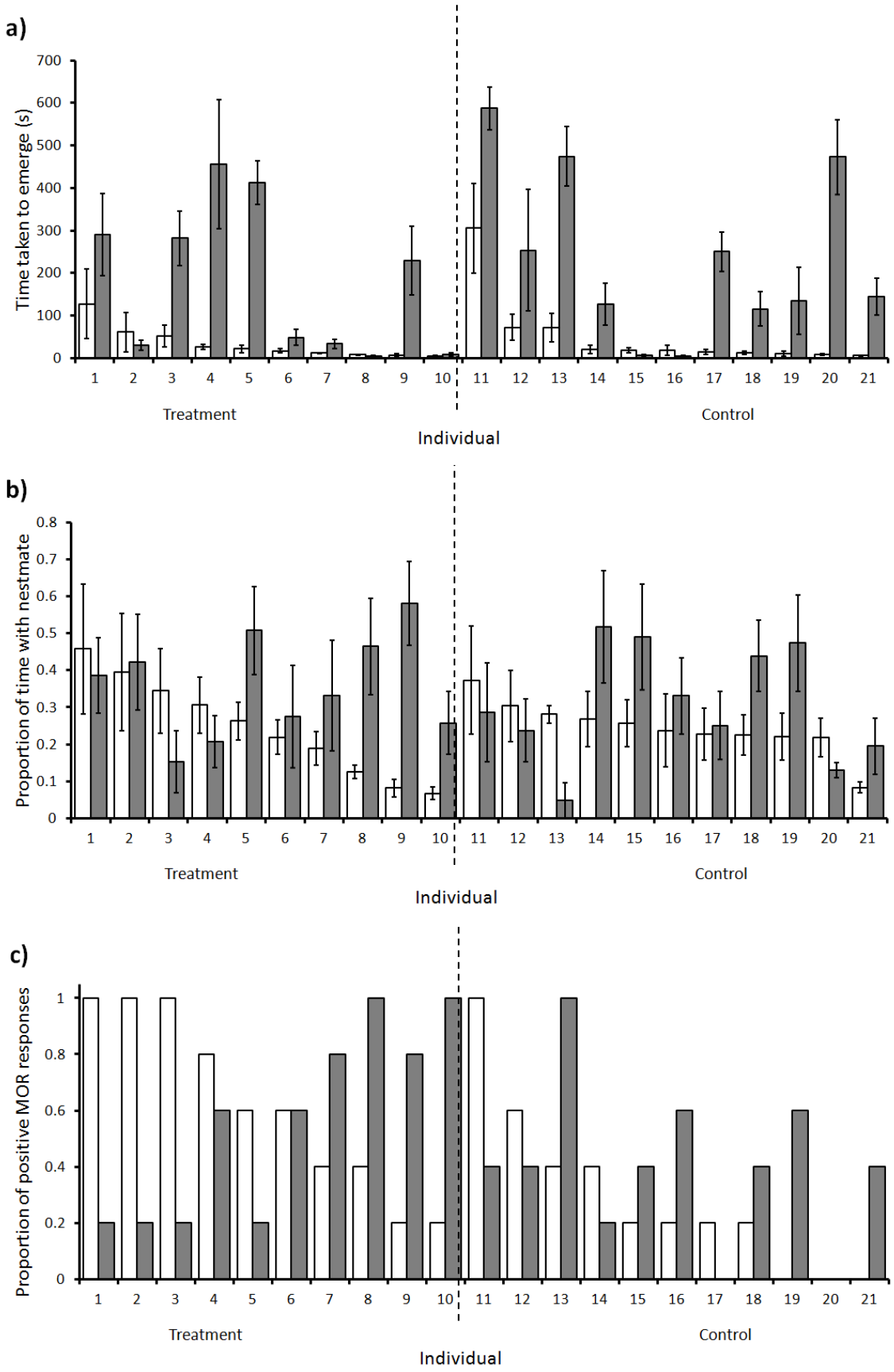


Figure 1 Effect of challenge with low dose of parasite The a) boldness, b) sociability and c) aggressiveness of *Formica rufa* wood ants either before (white columns) or after (grey columns) treatment with either a low dose (10^7 conidia/ml) of the *Metarhizium* parasite or a control solution, situated left and right of the dotted line respectively. Boldness is the mean ± s.e. time taken to emerge from a dark Petri dish, sociability is the mean ± s.e. proportion of assay time spent in proximity to a nestmate, and aggressiveness is the proportion of mandible opening responses (MOR) shown to a non-nestmate.

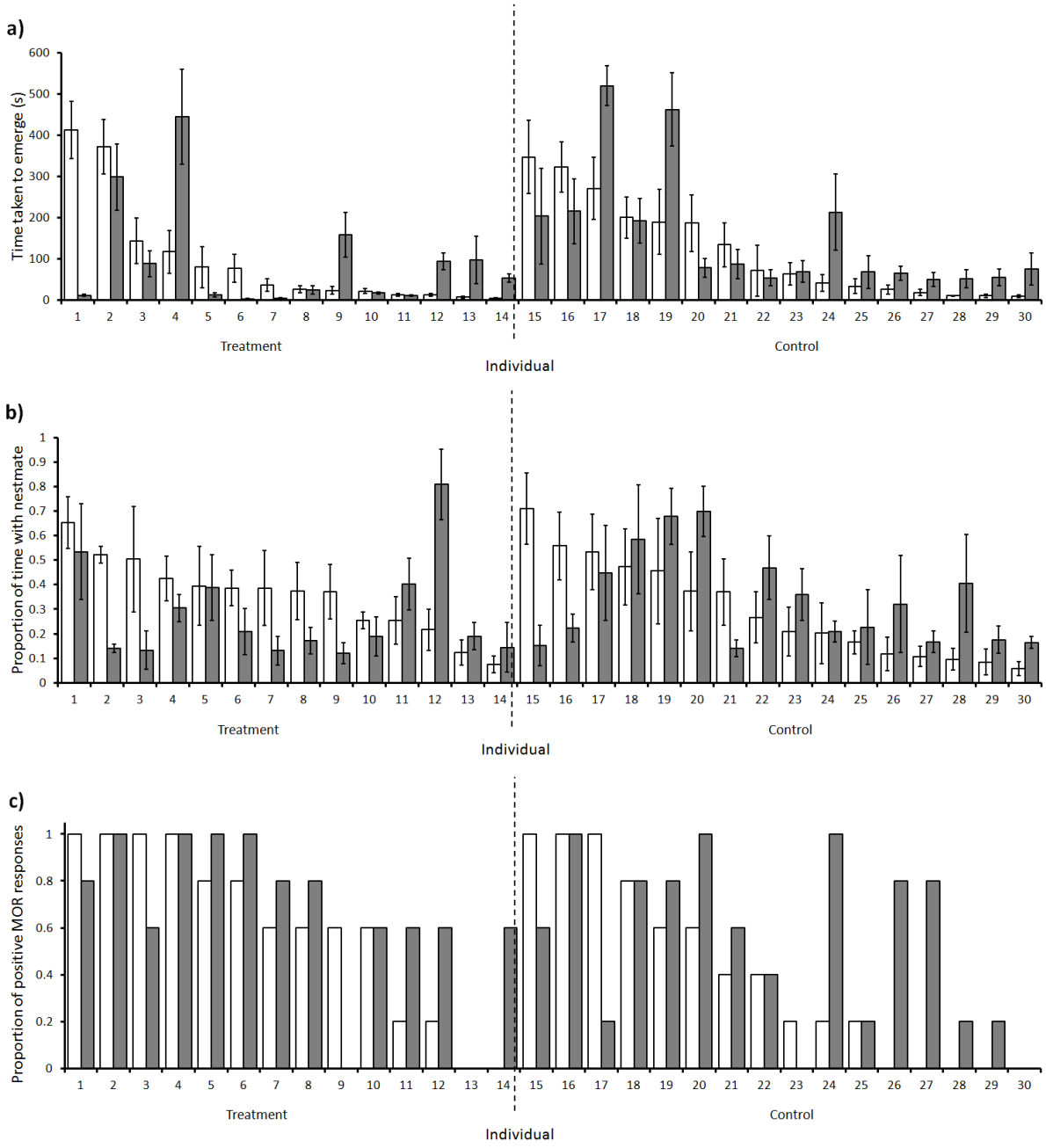
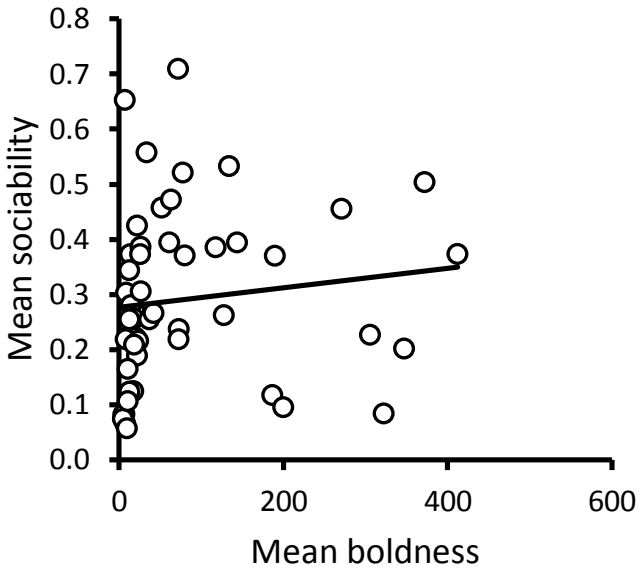
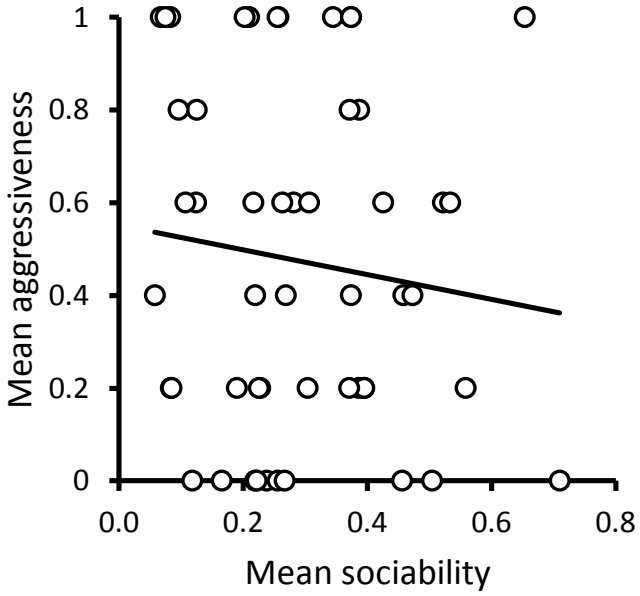


Figure 2 Effect of challenge with high dose of parasite The a) boldness, b) sociability and c) aggressiveness of *Formica rufa* wood ants either before (white columns) or after (grey columns) treatment with either a high dose (10^8 conidia/ml) of the *Metarhizium* parasite or a control solution, situated left and right of the dotted line respectively. Boldness is the mean ± s.e. time taken to emerge from a dark Petri dish, sociability is the mean ± s.e. proportion of assay time spent in proximity to a nestmate, and aggressiveness is the proportion of mandible opening responses (MOR) shown to a non-nestmate.





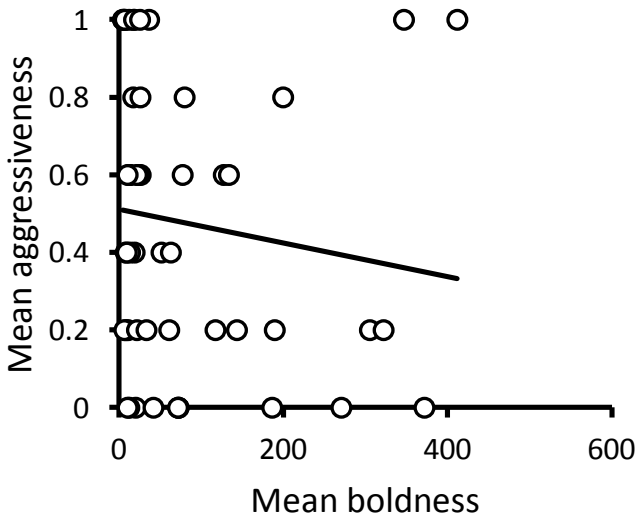


Figure 3 The correlations between the mean behavioural traits for each individual *Formica rufa* wood ant before treatment. Boldness is the time taken to emerge from a dark Petri dish, sociability is the proportion of assay time spent in proximity to a nestmate, and aggressiveness is the proportion of mandible opening responses (MOR) shown to a non-nestmate.

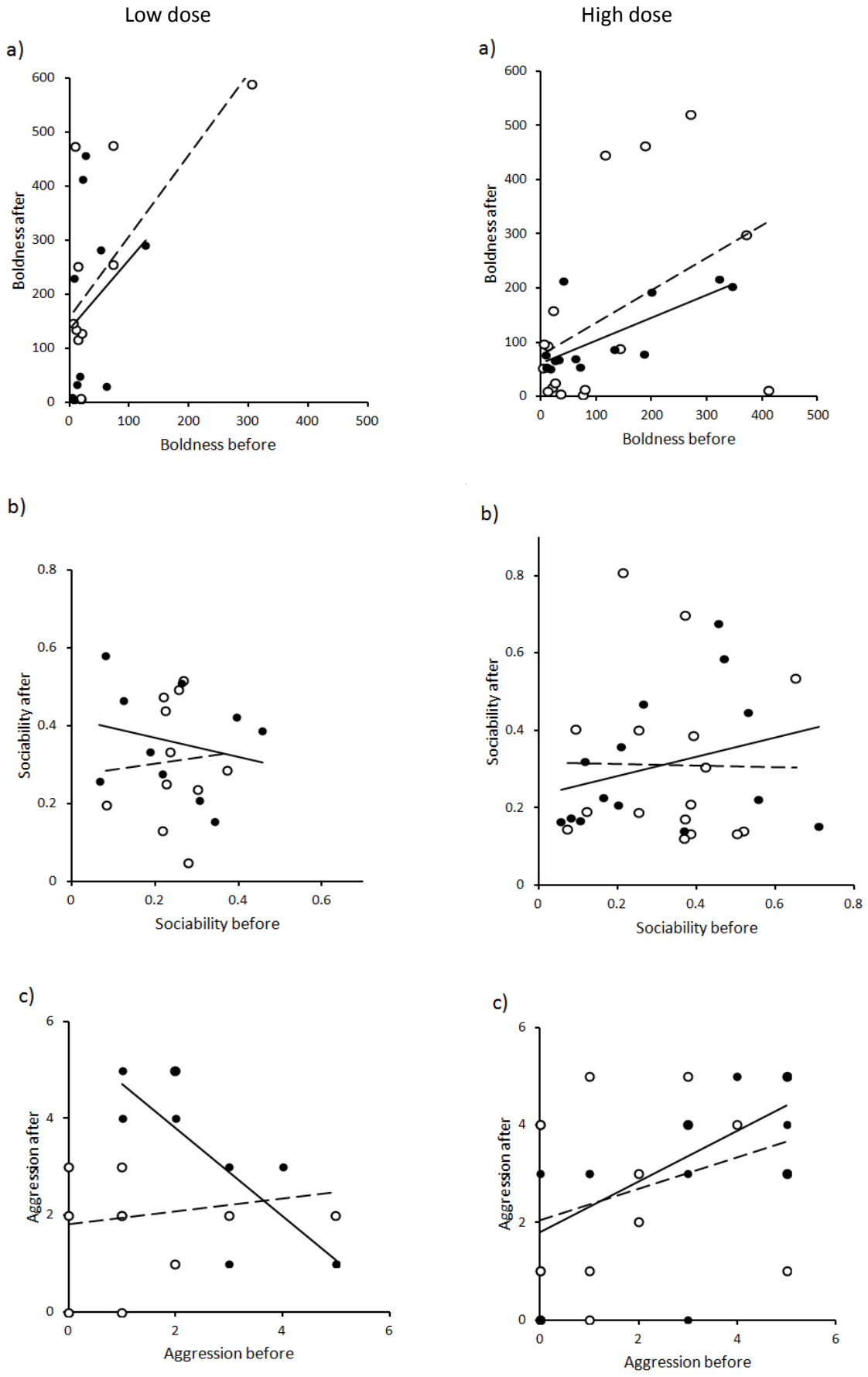


Figure 4 Relationships between the mean a) boldness, b) sociability and c) aggressiveness of *Formica rufa* wood ants 'before' and 'after' exposure to either the *Metarhizium* parasite (filled circles and solid line) or control solution (open circles and dashed line). Graphs on the left show the results of the low dose experiment; graphs on the right show the results of the high dose experiment. Boldness is the time taken to emerge from a dark Petri dish, sociability is the proportion of assay time spent in proximity to a nestmate, and aggressiveness is the proportion of mandible opening responses (MOR) shown to a non-nestmate.

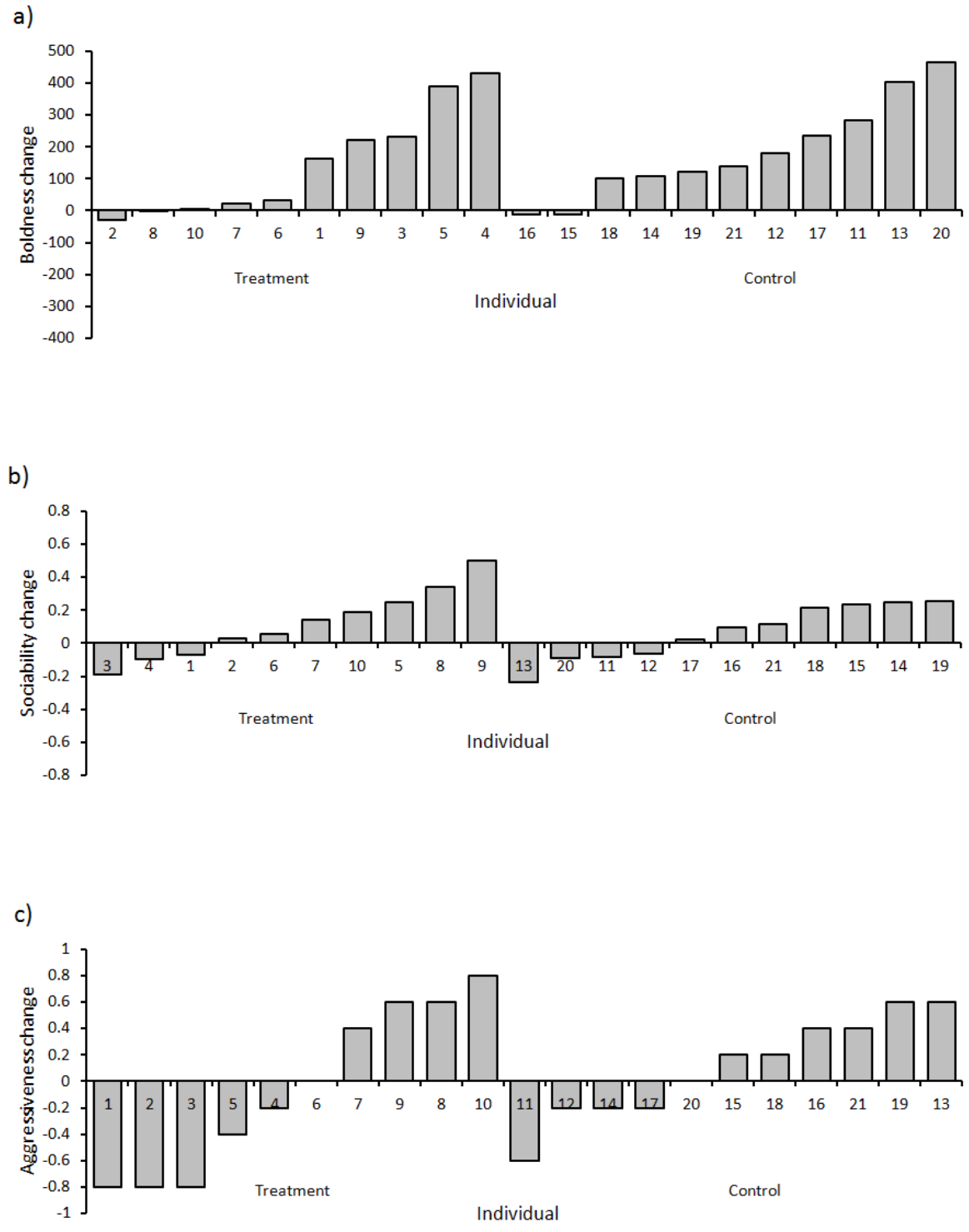


Figure 5 Effect of challenge with low dose of parasite The change in the mean a) boldness, b) sociability and c) aggressiveness of each individual *Formica rufa* between the 'before' and 'after' trials having either been challenged with a 1 x 10^7 conidia/ml suspension of the *Metarhizium* parasite ('Treatment') or a control solution ('Control') between trials.

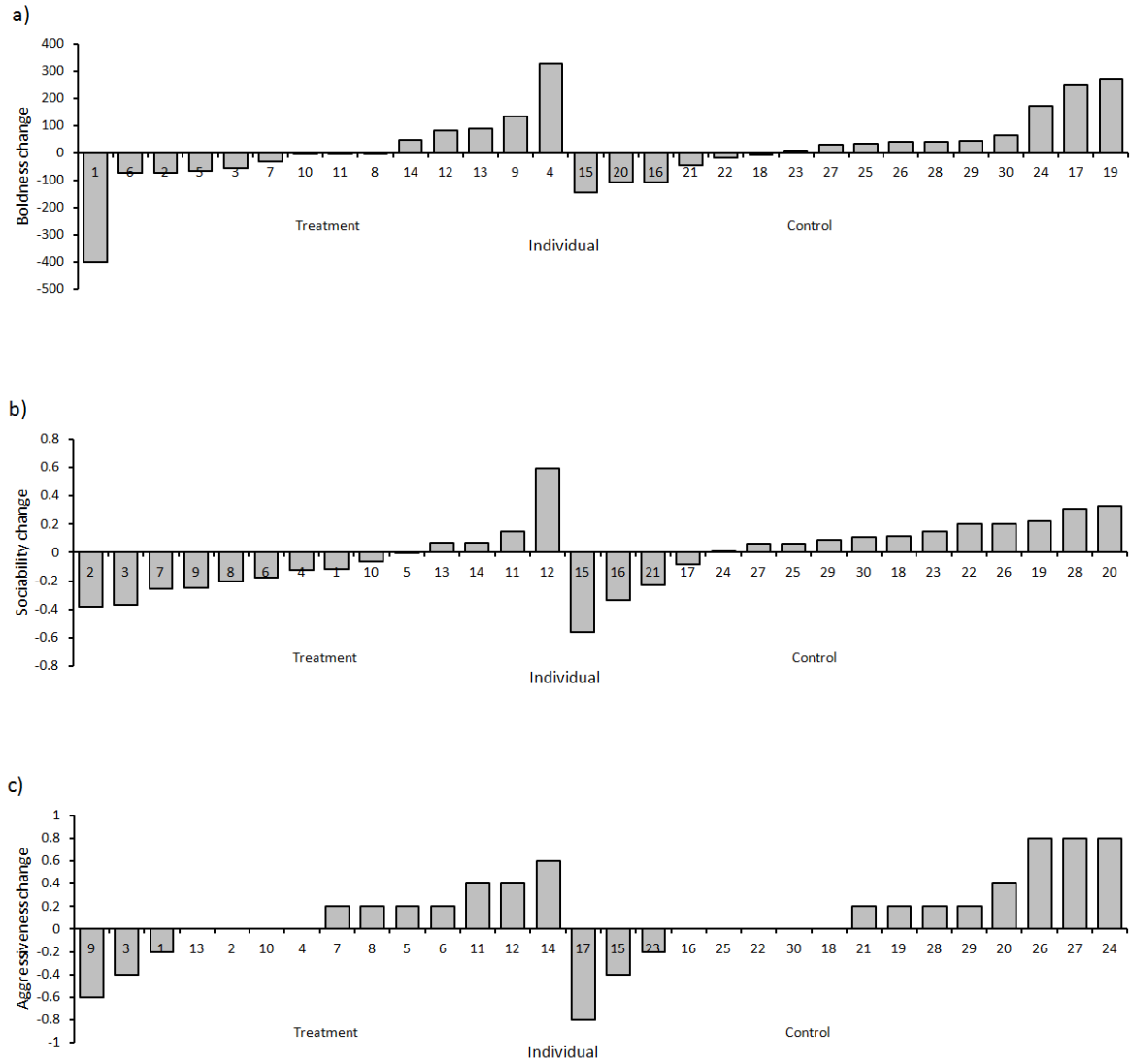


Figure 6 Effect of challenge with high dose of parasite The change in the mean a) boldness, b) sociability and c) aggressiveness of each individual *Formica rufa* between the 'before' and 'after' trials having either been challenged with a 1 x 10^8 conidia/ml suspension of the *Metarhizium* parasite ('Treatment') or a control solution ('Control') between trials.

Experiment 2: Fine time-scale experiment

The tendency of the ants to respond aggressively to a non-nestmate remained relatively constant until approximately 48 h after treatment, after which they tended to show a reduced aggressiveness (Fig. 7). At most time intervals the average aggressiveness score of each group was lower for the treatment group than the control group and the main effects of both time and treatment were significant (χ^2 = 18.8, df = 5, P = 0.002; χ^2 = 7.67, df = 1, P = 0.006), however there was no significant interaction between time and treatment (χ^2 = 7.67, df = 5, P = 0.606).

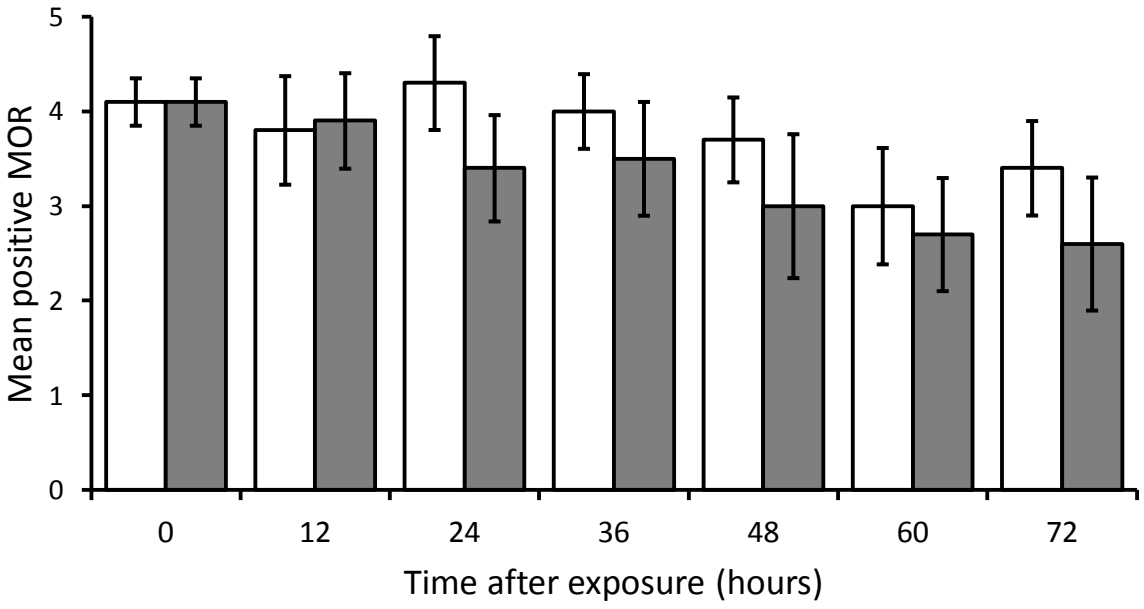


Figure 7 The mean ± s.e number of positive mandible opening responses of *Formica rufa* wood ants to a non-nestmate either treated with a 10^8 conidia/ml solution of the *Metarhizium* parasite (grey columns) or a control solution (white columns) at 12-hour time intervals. For the '0hrs' column all ants were untreated. For each column n = 10 individuals.

Experiment 3: Physiological stress experiment

Similarly to Experiment 1, ants showed significant individual differences in their tendency to respond aggressively to a non-nestmate, with some individuals responding aggressively on all occasions and others showing no aggressive responses (χ^2 = 551, df = 111, P < 0.001). On average individuals tended to respond to approximately one half of all stimuli in all treatment groups and this was similar before and after treatment (χ^2 = 2.28, df = 1, P = 0.131) for all treatments (χ^2 = 8.95, df = 4, P = 0.062) (Fig. 8). There was no significant interaction between treatment and trial (χ^2 = 2.83, df = 4, P = 0.587), indicating that none of the treatments significantly affected the ants' behaviour.

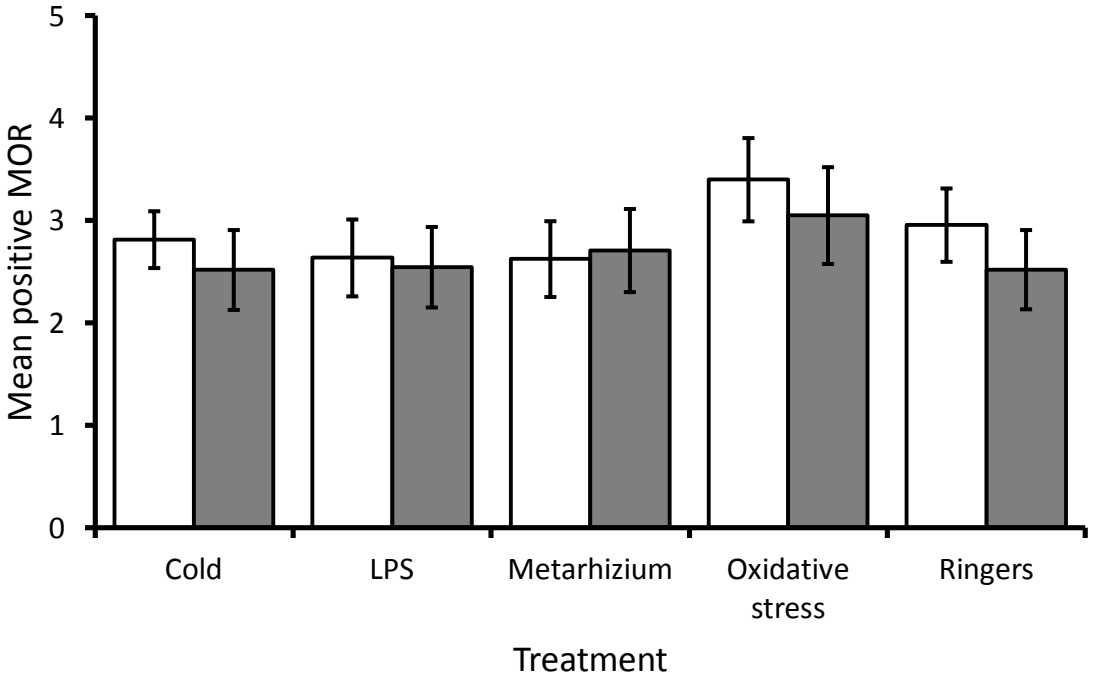


Figure 8 The mean ± s.e number of positive mandible opening responses of *Formica rufa* wood ants to a non-nestmate either before (white columns) or after (grey columns) exposure to various treatments. For the 'Cold' treatment ants were freeze-shocked at -18°C for 3 minutes (n = 27 individuals); for the 'LPS' treatment ants were injected with lipopolysaccharide to stimulate an immune response (n = 22 individuals); for the '*Metarhizium'* treatment ants were challenged with 10^8 conidia/ml solution of the *Metarhizium* parasite (n = 24 individuals); for the 'Oxidative stress' treatment ants were injected with paraquat to stimulate oxidative stress (n = 20 individuals); for the control 'Ringer's' group ants were injected with Ringer's solution (n = 23 individuals).

Discussion

Previous work on other, primarily non-social, species has found that animals commonly exhibit consistent individual variation in their behaviour, and it may be that this variation (known as animal personality) can change in various ways due to infection with parasites. The results of this study demonstrate that the wood ant *Formica rufa* shows similar individual variation in a number of behavioural traits and that this variation can be quite large within a colony, although there was little evidence of any correlations between these personality traits (behavioural syndromes). There was no consistent effect of *Metarhizium* on the ants' personalities, and in most cases the ants reacted similarly to the control and treatment conditions; yet certain assays appeared to show that the disease does play a role in modifying behaviour.

The results of Experiment 1 provide evidence for the existence of personality in *Formica rufa*, which can be considered an additional example of within-colony behavioural variation in a social insect to add to the growing list of others (e.g. see Jandt *et al*., 2013). The existence of animal personality is now well established in the animal behaviour literature (Sih *et al*., 2004; Bell, 2007b), with numerous studies attempting to investigate both its proximate genetic and physiological causes and its evolutionary significance (Wolf & Weissing, 2012; Biro & Stamps, 2008). The presence of consistent variations in behaviour among social insects is unsurprising given their intra-colonial phenotypic polymorphism, in many species taking the form of separate, distinct castes with morphological and behavioural specialisations (Hölldobler & Wilson, 1990). However, individual behaviour can also vary consistently within these divisions and individuals can vary in their behavioural traits within castes (Jandt *et al*., 2013). There is evidence that much of the variation in behaviour within a particular group or colony may be due to the most extreme (i.e. most aggressive, bold etc)

individual within the group (Pruitt *et al*., 2013), suggesting that individual personalities may differ greatly even in a specialised group. *Formica rufa* are relatively monomorphic compared to other ant species with advanced division of labour (Wilson, 1953), typically ranging between 4.9 and 12.9mg (Hurlbert *et al*., 2008), yet personality differences may play a role in task allocation as is the case in *Myrmica rubra* (Pamminger *et al*., 2014). Such variation could be adaptive for the ants given that variation in behavioural traits such as aggression can increase the fitness of the colony, as compared to colonies with more consistent behaviour (Modlmeier & Foitzik, 2011). This is speculated to be because colonies composed of more similar individuals may suffer certain costs; for instance a colony of uniformly aggressive members may gain a predation advantage but also be susceptible to between-nestmate aggression and diminished colony cohesiveness (Pruitt & Riechert, 2011).

However, unlike other studies on animal personality, the evidence for correlations between these personality traits was limited. Previous work has shown that separate personality traits are frequently correlated with each other, particularly aggression and boldness, which has been observed in many species such as stickleback fish (Bell & Stamps, 2004; Bell & Sih, 2007) and notably *Myrmica* ants (Chapman *et al*., 2011). The *F. rufa* tested in this study lacked this particular syndrome and showed only a weak correlation between boldness and sociability. The difference in the overall behavioural variation between *F. rufa* and these other species could be due to a variety of reasons such as their different habitats and life history strategies (Carter *et al*., 2013). Behaviours can be correlated with each other and with other phenotypic traits in numerous complex ways; for instance while individual boldness and aggression are highly correlated in the social spider *Stegodyphus sarasinorum*, various components of its prey capture behaviour are predicted by boldness but not aggression, as well as by other traits like age, body size and dispersal distance that are themselves unrelated to both boldness and aggression (Grinsted *et al*., 2013). Thus it may be the case that

aggression is displayed by *F. rufa* in different contexts to other species and does not relate to boldness. Additionally, since the ants were sampled randomly from multiple colonies and without specific consideration for body mass or worker location/task differentiation, the lack of evidence for substantial behavioural syndromes at the species- or metapopulation-level may be confounded by syndromes at other scales such as colony or task-allocation, and further work could investigate this.

Despite Experiment 1 confirming the existence of personality in *F. rufa*, the impact of disease on this personality is less apparent from the results. The boldness assay has the clearest results: Individuals that took similar amounts of time to emerge from a refuge (i.e. were of similar boldness) before treatment tended to take less time to emerge from a refuge (i.e. were bolder) after being challenged with *Metarhizium* than those that were not exposed to the fungus. Likewise, ants challenged with a high dose of *Metarhizium* conidia appear to become less sociable to a marginally greater extent than untreated ants, although there was no comparable trend in ants challenged with the lower dose. In the case of aggressiveness, exposure to the fungal parasite had no discernible impact on the ants' tendency to respond aggressively to a non-nestmate. These observations suggest that parasites may affect the average personality across populations and the consistency of behavioural traits over time, but that overall *F. rufa* personality is rather resilient to the effects of parasite infection.

These findings are somewhat different to other studies such as Bos *et al*. (2012), who found that carpenter ants infected with *Metarhizium* became considerably less sociable, engaging in less trophyllaxis activity and in the later stages of infection associating less with brood and often leaving the nest, which may be an adaptation by the ants to minimise the spread of infection within the colony; but also found that ants engaged in more aggressive behaviour against non-nestmates. It is speculated that this may also be adaptive given the ants infected with the obligate killer fungus have a limited lifespan and will be more likely to risk

aggressive ‘sacrificial’ behaviours in defence of the colony. Another suggested explanation is that in a species with age-dependent polyethism infection may act as a form of ageing, causing ants to prematurely transition to more aggressive foraging behavioural types. However, the data presented here, while showing a similar but small effect on sociability at high concentrations of conidia, show instead that the aggressiveness of *F. rufa* is unaffected by *Metarhizium*. One possible explanation is that the challenged ants did not actually become infected because wood ants have many defences against infection (Marikovsky, 1962; Chapuisat *et al.*, 2007; Aubert & Richard, 2008), but the ants were isolated after having been challenged and could not have benefitted from social immunity. Alternatively it may be that the conidia on the ants’ cuticles did not successfully germinate and infect the ants but the experiment tested the ants at high doses that are known to be lethal (Qiu *et al.*, 2014) and killed the majority of ants challenged (see Appendix).

The amount of time since infection could have had an effect on the results because the stage of infection may correspond to different behavioural modifications. Germination of *Metarhizium* conidia, penetration of the cuticle and establishment of infection can take 2-4 days (Gillespie *et al.*, 2000; Bos *et al.*, 2012), and it is conceivable that either parasitic modification of ant behaviour or ants’ behavioural defences against parasitism would manifest themselves differently in the later stages of infection compared to the earlier stages. The results of Experiment 2 show that the aggressiveness of *F. rufa* began to decline after 48 hours from the time of exposure to *Metarhizium*, however this decline occurred in both the treatment and control groups, indicating that the reason for the decrease was not due to the presence of the parasite and may have been due to other factors. Removing a social insect from the colony is known to cause it stress, as is the case in honeybees (Seehuus *et al.*, 2005), and it is known that social isolation can affect animal personality (Lihoreau *et al.*, 2009). It may be that isolating each ant for the purposes of the experiment caused their behaviour to

gradually change over time. This raises the question of whether other physiological effects may have a greater impact on behaviour than infection, which was investigated in Experiment 3. The results, however, show that while the ants' aggressiveness tended to decline in the 48 hours between trials, similarly to the other experiments, none of the stressors had a significantly different effect. Thus neither cold-shock, oxidative stress, immune activation nor parasite challenge had an additive impact on the aggressiveness of *F. rufa* beyond whatever decline the ants tended to experience between trials, providing evidence that their personality differences remain quite consistent even in response to quite strong periods of stress.

This study provides evidence of personality in an additional invertebrate species, the list of which is currently rather small (Kralj-Fišer & Schuett, 2014). Furthermore, the study shows that overall personality is remarkably resilient to the effects of parasites and other stresses, even in the case of the lethal parasite infections tested here. It may be that in social insects personality is particularly resistant to the effects of infection because they are part of a eusocial superorganism (Hölldobler & Wilson, 2009; Hughes, 2012), gaining their fitness predominantly or entirely indirectly by ensuring the reproductive success of their relatives. The importance of behavioural variation at higher levels of organisation in social insects could mean that from the point of view of parasites attempting to maximise their transmission or colonies as a whole attempting to prevent infection, individual behaviour has less impact, and stronger effects of parasite infection on personalities may be seen in solitary animals.

Chapter 3 Concluding Discussion

The results described in this thesis indicate that the ant species *Formica rufa* have individual personalities, which are resilient towards numerous external and internal stresses. One of these – disease – is significant because parasites are known to commonly influence animal behaviour in a variety of ways (Moore, 2002; Poulin, 2010; Thomas *et al*., 2010), suggesting that certain behavioural tendencies are maintained even in the face of severe physiological pressures. Parasitic manipulation, whereby parasites alter host behaviour to increase their own transmission, is a particularly noticeable example of the influence disease can have (Thomas *et al*., 2005). However, these effects, either as an adaptive alteration by the parasite or a coincidental byproduct of the infection process, appear to be nonexistent in the host-parasite system in the present study. Furthermore, the absence of a consistent change in behaviour in this species, a social insect, may have implications for understanding the way behaviour of individuals responds to environmental factors and shapes the behaviour of colonies in social species. By confirming the existence of personality in a social insect and exploring the lack of effect of a fungal parasite on these individual differences, this thesis is a step towards combining sociobiology with the study of the effect of parasites on behaviour.

The lack of considerable effect of parasitism on ant personality could be investigated further to clarify the results in this thesis. For instance, the study considered the average behavioural scores of the individual ants from the repeats of the assays in the different trials, yet an potential alternative effect of the fungus could be to increase the heterogeneity of behavioural traits (Poulin, 2013), which would affect the behaviour of the group while leaving the mean individual personalities the same. The study could be expanded by using other species of

fungi, incorporating generalist and specialist pathogens. Additionally a broader range of personality traits could be used, and the reliability of the measurements could be increased by quantifying the traits in more than one way, using multiple assays that show 'convergent validity' (tests for the same personality trait that are in fact measuring the same thing) and 'divergent validity' (tests for different traits that are not simply measuring the same trait in different ways) (Carter *et al*., 2013). To do so it will be necessary to compare a wide range of interrelated behavioural traits, however these should be selected carefully in order to test specific hypotheses rather than selecting the maximum number of measurable behaviours possible (Poulin, 2013). It would also be interesting to determine whether any potential effect of parasites on behaviour that does exist is adaptive. Personality traits can be advantageous; for example boldness, activity and aggression may be linked to greater productivity (Biro & Stamps, 2008), and parasitic manipulation could be a way of transferring the benefit to the parasite. Future work could delve into these potential effects by comparing the behavioural syndromes of non-infected and experimentally-infected colonies and looking at colony survival and fitness.

Another direction would be to compare species of social insects at different levels of sociality. Insect societies exhibit a gradient from subsocial to eusocial and some species have more limited division of labour (Wilson, 1971; Hölldobler & Wilson, 1990). In these cases the usefulness of studying colony-level personalities is likely to be more limited because castes will be less differentiated and also the colony experiences more internal conflict between queens and workers over egg production and resource allocation. However, even in this case the colony will act in a coordinated way for some tasks like resource acquisition, and this may still provide the opportunity to investigate colony-level behavioural differences in infected and non-infected individuals. Also, species in which workers show temporal switches in task (e.g. older honeybees engaging in more hygienic behaviour [Wilson-Rich *et*

al., 2009]), known as temporal polyethism, have less consistent division of labour. These shifts are interesting because they may occur alongside physiological changes mediated by genetic or environmental factors, and physiological differences can be linked to behavioural effects such as cold resistance in ants (Modlmeier *et al*., 2012b). Therefore they could be investigated and compared with any parasite-induced changes in behaviour to help work out the underlying processes involved in behavioural change.

Increased knowledge of personality differences and effect of parasites on behaviour, from studying both the mechanisms using increasingly sophisticated and easily available molecular techniques like proteomics (Hughes, 2013), and the ecological and evolutionary dynamics involved, will help to deepen the understanding of animal behaviour and disease biology. Experiments along the lines of some of the ideas set out above could provide some enlightening results to enrich those presented in this thesis. As other authors have optimistically expressed, such findings will continue to be reinforced and/or revaluated in light of ongoing insights into the genetic and molecular basis of animal behaviour with the hope of forming a better picture the ways in which host behaviour is influenced by disease.

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Appendix

Formica rufa survivorship

The survivorship of *Formica rufa* treated with *Metarhizium anisopliae* was investigated to verify that the parasite was successfully infecting a substantial proportion of the individuals tested. Ants were treated with 1μL of a *Metarhizium* conidia suspension on the outside of their abdomens. Three concentrations were used: 1 x 10^7 conidia/ml, 5 x 10^7 conidia/ml and 1 x 10^8 conidia/ml. 1μL of 0.05% Triton X solution was used as a control. Individuals were then returned to their plastic pots and provided with cotton wool soaked in water and 10% sucrose solution. The survivorship of the ants was checked at 24 h intervals. 10 ants were tested in each treatment group, giving a total sample size of 40.

Table A1 and Figure A1 below show the mortality of the ants treated with the different concentrations of fungal conidia over 15 days.

Table A1 The survival of *Formica rufa* treated with suspensions of conidia of the parasite *Metarhizium anisopliae* at different concentrations

	Proportion surviving														
Time(days)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Dose (conidia/ml)															
0	1	1	1	1	1	1	1	1	1	0.9	0.9	0.9	0.9	0.7	0.7
1 x 10^7	1	0.9	0.9	0.9	0.9	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.5
5 x 10^7	1	0.8	0.8	0.8	0.8	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3
1 x 10^8	1	0.9	0.9	0.8	0.8	0.7	0.7	0.7	0.7	0.7	0.7	0.5	0.5	0.4	0.3

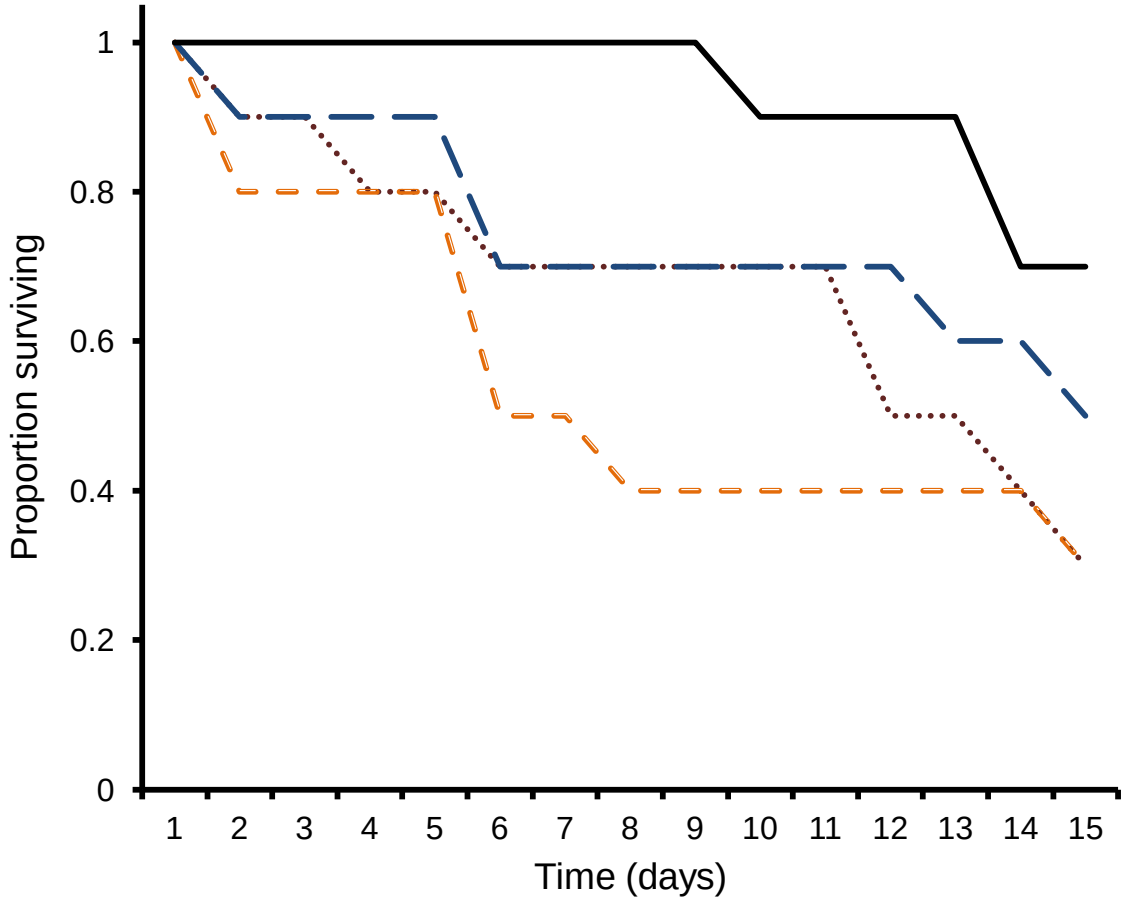

Figure A1 The survival of *Formica rufa* treated with suspensions of conidia of the parasite *Metarhizium anisopliae* at different concentrations: 1 x 10^7 conidia/ml (broad-dashed line), 5 x 10^7 conidia/ml (narrow-dashed line) and 1 x 10^8 conidia/ml (dotted line), and a control solution (solid line).